Tropicibacter multivorans sp. nov., an aerobic alphaproteobacterium isolated from surface seawater

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Strain MD5^T, an aerobic marine alphaproteobacterium, was isolated from Mediterranean seawater at Malvarrosa beach, Valencia, Spain. The strain was characterized in a polyphasic study and was placed phylogenetically within the *Roseobacter* clade in the family *Rhodobacteraceae*. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain MD5^T is related to *Tropicibacter naphthalenivorans* C02^T, *Phaeobacter inhibens* T5^T, *P. gallaeciensis* BS107^T and *P. daeponensis* TF-218^T, with 96.9, 96.2, 96.1 and 96.1 % sequence similarity, respectively. Phylogenetic analyses also showed that strain MD5^T forms a stable clade only with *T. naphthalenivorans* C02^T. Strain MD5^T requires Na⁺ plus a divalent cation (either Mg²⁺ or Ca²⁺) to grow, does not reduce nitrate to nitrite and uses a large number of carbohydrates as sole carbon sources. It is positive for β -galactosidase and urease activities and aesculin hydrolysis. Enzyme activities displayed in the API ZYM strip were alkaline phosphatase, leucine arylamidase, acid phosphatase and α -glucosidase. Major cellular fatty acids were summed feature 8 (C_{18:1} ω 7c and/or C_{18:1} ω 6c; 70.9 %) and C_{16:0} (8.2 %). The results of physiological and biochemical tests allowed clear phenotypic differentiation of this isolate from the only described species of the genus *Tropicibacter*. It is evident from the genotypic and phenotypic data obtained that the strain should be classified in a novel species in the genus *Tropicibacter*. The name *Tropicibacter multivorans* sp. nov. is proposed, with the type strain MD5^T (=CECT 7557^T =KCTC 23350^T).

The family *Rhodobacteraceae* in the class *Alphaproteobacteria* of the phylum *Proteobacteria* mainly comprises bacteria of aquatic origin, many of them requiring NaCl for growth. Within this family, the so-called *Roseobacter* group is one of the most abundant and ubiquitous. Almost two decades since the description of *Roseobacter* (Shiba, 1991), this group has expanded to more than 50 genera, as can be seen in the last release (LTPs102_SSU) of the Living Tree Project database (Yarza *et al.*, 2010).

The genus *Tropicibacter* belongs to the family *Rhodobacteraceae* and currently comprises only one species, *Tropicibacter naphthalenivorans*, described on the basis of a single isolate from seawater collected at Semarang Port in Indonesia (Harwati *et al.*, 2009). This marine bacterium was capable of degrading hydrocarbons, specifically naphthalene, as its name indicates.

A recently isolated strain, MD5^T, obtained from coastal seawater of the western Mediterranean Sea, has been characterized as a novel member of the genus *Tropicibacter* that shows the highest 16S rRNA gene sequence similarity to *T. naphthalenivorans*.

Strain MD5^T was isolated from surface seawater collected at Malvarrosa Beach (39° 28' 29'' N 0° 19' 23'' W; temperature 25 °C; pH 8) in Valencia, Spain, in July 2008. Seawater was plated on 1/100-strength marine agar (MA) plates [0.04 % marine broth (MB), 1.2 % bacteriological agar and aged, filtered seawater to maintain the salts concentration]. Incubation was done at 26 °C over the course of several days, with periodic examination and isolation of new colonies. Isolated colonies were streaked on the same medium and subcultured under the same conditions until pure cultures were obtained. Strain MD5^T was isolated after 4 days of incubation and was maintained as cell suspensions in MB plus 20 % glycerol at –80 °C. Strain MD5^T was grown routinely on MA and in MB at 28 °C for 24–48 h to prepare inocula. Media other than MA and MB were supplemented with half-strength artificial seawater (ASW;

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MD5^T is FR727679.

Five supplementary figures and a supplementary table are available with the online version of this paper.

400 mM NaCl, 100 mM MgSO₄·7H₂O, 20 mM KCl and 20 mM CaCl₂·2H₂O) or marine cations supplement (MCS; Farmer & Hickman-Brenner, 2006). Suspension media for microscopic observations or miniaturized testing were also prepared using salt-supplemented solutions. The phenotypic characteristics of the strain were determined following methods described previously (Macián *et al.*, 2001) for microscopic observation of cells, colony characteristics on MA, oxidase, catalase, nitrate reduction, fermentation of D-glucose in O/F medium, temperature range for growth (4, 15, 28, 37, 40 and 45 °C), hydrolysis of gelatin, casein, starch, lecithin, alginate, Tween 80 and DNA and use as sole carbon sources of 26 carbohydrates, 12 organic acids and 16 amino acids and amines on basal medium agar [BMA; 50 mM Tris/HCl, pH 7.5; 19 mM NH₄Cl; 0.33 mM K₂HPO₄·3H₂O; 0.1 mM FeSO₄·7H₂O and 1.3 % (w/v) purified agar (Oxoid) on half-strength ASW; Baumann & Baumann, 1981].

API ZYM, API 20NE (bioMérieux) and GN2 (Biolog) tests were performed in duplicate. For comparative purposes, *T. naphthalenivorans* CECT 7648^T was tested in parallel as a reference.

Cell morphology, motility and flagella arrangement were observed on wet mounts under phase-contrast microscopy. Cells of strain MD5^T were small, straight, regular bacilli, 0.5–1 µm wide and 2–2.5 µm long, motile by polar flagella, as seen after flagella staining with the method of Heimbrook *et al.* (1989). In our laboratory, we were not able to see the peritrichous flagella reported by Harwati *et al.* (2009) in the description of *T. naphthalenivorans*. Cell size was determined by using electron microscopy at the Servicio Central de Soporte a la Investigación Experimental (University of Valencia). Samples were examined in a Hitachi S-4100 field emission scanning microscope with a 7–15 mm working distance and at an acceleration voltage of 10 kV. In addition, samples were examined using a transmission electron microscope (JEM-1010; JEOL) at 60 kV after negative staining with 2 % (w/v) phosphotungstic acid at an appropriate pH. A selection of images is given in Fig. S1, available in IJSEM Online.

The strain stained Gram-negative and cells lysed readily when suspended in 3 % (w/v) KOH solution. Cells grown for 5 days on MA or BMA plus citrate did not reveal polyhydroxybutyrate granules after staining with Nile blue A (Smibert & Krieg, 1994).

Growth of MD5^T on MA (28 °C, 48 h) yielded beige, brilliant and slightly umbonate colonies. Isolated colonies of strain MD5^T were about 1 mm in diameter with entire edges (MA, 28 °C, 48 h). The ability to grow at different temperatures was tested on MA incubated at 4 and 15 °C (for 15 days) and 28, 37, 40 and 45 °C (for 48 h). The strain was able to grow at 15–37 °C, but not at 4 or 40 °C. Ion requirements were tested on salt tolerance agar (STA), containing 1 % peptone, 0.3 % yeast extract and 1.2 % purified agar. This medium was supplemented with the following salt combinations (w/v): with (i) and without (ii) 2 % NaCl, (iii) with 2 % KCl, (iv) with 2 % NaCl and 0.9 %

MgCl₂·6H₂O, (v) with 2 % NaCl and 0.2 % CaCl₂·2H₂O, (vi) with 2 % NaCl and 0.06 % KCl, (vii) with 2 % NaCl, 0.9 % MgCl₂·6H₂O and 0.2 % CaCl₂·2H₂O and (viii) with 2 % NaCl, 0.9 % MgCl₂·6H₂O, 0.2 % CaCl₂·2H₂O and 0.06 % KCl. Strain MD5^T required Na⁺ plus a divalent cation (either Mg²⁺ or Ca²⁺) for growth, whereas *T. naphthalenivorans* CECT 7648^T required specifically Na⁺ plus Mg²⁺ and did not show any growth with Na⁺ plus Ca²⁺. Both strains were unable to grow with only Na⁺, only K⁺ or with these two cations combined. The salinity range for growth was tested on MA as reported previously (Macián *et al.*, 2005). Strain MD5^T showed good growth from 0.35 to 7 % (w/v) total salinity and no growth was observed at 8 % salinity or higher. *T. naphthalenivorans* CECT 7648^T showed the same minimum and maximum salinities for growth, in contrast with its original description, which reported its ability to grow with up to 15 % salts.

Strain MD5^T was oxidase- and catalase-positive, unable to ferment glucose on O/F medium and unable to reduce nitrate to nitrite (or gas) either in nitrate broth or API 20NE strips. In our laboratory, *T. naphthalenivorans* CECT 7648^T was oxidase- and catalase-positive and unable to reduce nitrate to nitrite, in disagreement with the original description of the genus *Tropicibacter* (Harwati *et al.*, 2009), defined as positive for nitrate reduction.

Results for strain MD5^T in API 20NE, API ZYM and Biolog GN2 kits are reported in the species description. Strain MD5^T and *T. naphthalenivorans* CECT 7648^T were unable to oxidize any of the substrates contained in Biolog GN2 or gave weak responses. This is in sharp contrast with the description of *T. naphthalenivorans* (Harwati *et al.*, 2009), where strain C02^T was reported as able to metabolize a large number of substrates in this system.

Fatty acid methyl esters were extracted and prepared at the CECT according to standard protocols described for the MIDI Microbial Identification System (Sasser, 1990). Cellular fatty acid content was analysed by GC with an Agilent 6850 chromatographic unit, with the MIDI Microbial Identification System using the RTSBA6 method (MIDI, 2008) and identified using the Microbial Identification Sherlock software package. Strain MD5^T, *T. naphthalenivorans* CECT 7648^T, *Phaeobacter inhibens* CECT 7251^T, *P. gallaeciensis* CECT 7277^T, *P. daeponensis* CECT 7309^T, *Shimia marina* CECT 7688^T and *Nautella italica* CECT 7645^T were cultured on MA and incubated at 28 °C for 48 h. The predominant fatty acid constituent of MD5^T and *T. naphthalenivorans* CECT 7648^T was summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c). However, the fatty acid profile of strain MD5^T showed a distinctive pattern, allowing its differentiation from *T. naphthalenivorans* CECT 7648^T. Strain MD5^T possessed fatty acids C_{10:0} 3-OH, C_{12:0} 3-OH and C_{16:0} 2-OH but not C_{12:1} 3-OH or C_{18:1}ω9c (Table 1).

Polar lipid analysis of strain MD5^T and *T. naphthalenivorans* CECT 7648^T was performed at the CECT as follows. Polar lipids were extracted from 100 mg freeze-dried cells and separated by two-dimensional silica-gel TLC (Tindall *et al.*,

Table 1. Cellular fatty acid compositions of strain MD5^T and related type strains

Strains: 1, *T. multivorans* sp. nov. MD5^T; 2, *T. naphthalenivorans* CECT 7648^T; 3, *Phaeobacter inhibens* CECT 7251^T; 4, *P. gallaeciensis* CECT 7277^T; 5, *P. daeponensis* CECT 7309^T; 6, *Shimia marina* CECT 7688^T; 7, *Nautella italica* CECT 7645^T. All data were obtained in this study (mean values of at least three replicates) using strains grown on MA for 48 h at 28 °C. Cellular fatty acid analysis was carried out according to the Microbial Identification System (Microbial ID). Fatty acids that gave only trace amounts or were not detectable in all strains are not represented. —, Not detected; tr, trace amount (<1 %).

Fatty acid	1	2	3	4	5	6	7
Hydroxy							
C _{10:0} 3-OH	4.8	—	2.4	2.6	2.5	9.5	2.9
C _{12:0} 3-OH	1.0	—	1.8	2.4	2.4	2.4	2.4
C _{12:1} 3-OH	—	3.4	—	—	—	—	—
C _{16:0} 2-OH	3.8	—	1.7	3.4	3.1	—	1.2
C _{18:0} 3-OH	—	—	—	—	—	1.1	—
Saturated							
C _{9:0}	tr	1.5	—	tr	—	—	—
C _{12:0}	tr	tr	tr	tr	0.9	1.0	tr
C _{14:0}	0.9	2.1	tr	0.9	tr	tr	tr
C _{16:0}	8.2	7.4	7.2	9.0	2.3	6.2	4.0
C _{18:0}	2.7	6.9	1.7	3.3	1.0	1.9	1.2
iso-C _{18:0}	—	—	—	tr	3.8	—	4.7
Unsaturated							
11-Methyl C _{18:1ω7c}	5.3	1.8	8.6	8.1	9.7	2.2	1.3
C _{18:1ω9c}	—	1.3	—	—	—	—	—
Summed feature 8*	70.9	73.5	73.7	68.0	71.6	69.5	81.0

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed feature 8 contained C_{18:1ω7c} and/or C_{18:1ω6c}.

2007). Total lipid material was stained with molybdatophosphoric acid followed by heating at 150 °C for 15 min. For the visualization of specific functional groups, α-naphthol/sulphuric acid, ninhydrin, molybdenum blue and Dragen-dorff's reagent were used, as described previously (Tindall *et al.*, 2007). The two strains showed very similar profiles, and the major identifiable components were phosphatidyl-ethanolamine, phosphatidylglycerol and phosphatidylcholine (Fig. S2).

Analysis of respiratory quinones was carried out by the Identification Service of the DSMZ and Dr B. J. Tindall (DSMZ). Strain MD5^T had a ubiquinone system (Q-10) that was in agreement with reported data for other members of the *Roseobacter* clade.

Sample preparation for matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) protein analysis was carried out at the CECT according to the ethanol/formic acid extraction protocol recommended by Bruker Daltonics (<http://www.bdal.de>). MALDI-TOF MS

was conducted using a Reflex IV mass spectrometer (Bruker Daltonics) equipped with a N₂ laser. All spectra were recorded in linear positive-ion mode. The acceleration voltage was 20 kV. Spectra were collected as a sum of 240 shots across a spot, and 12–16 spots per strain. A mass range of 2000–20 000 Da was used for analysis. Peak recognition and comparison of the spectra were done with the BioTyper software 1.1 as described by Maier *et al.* (2006). The dendrogram obtained with MALDI-TOF MS illustrates that strain MD5^T and *T. naphthalenivorans* CECT 7648^T can be separated clearly from each other and from members of the related genera *Phaeobacter*, *Nautella* and *Shimia* (Fig. S3).

The DNA G+C content was determined by the Identification Service of the DSMZ by HPLC according to the method described by Tamaoka & Komagata (1984) and Mesbah *et al.* (1989) as 60.7 mol%.

The almost-complete 16S rRNA gene sequence of strain MD5^T was obtained in this study. A cell pellet was collected and DNA was extracted using a microbial DNA isolation kit (Ultra Clean; Mo Bio Laboratories). Amplification and partial sequencing of the 16S rRNA gene was performed as described previously (Lucena *et al.*, 2010). In parallel, a stretch of around 1000 nt close to the 5' end of the 16S rRNA gene was sequenced for strain *T. naphthalenivorans* CECT 7648^T as an authenticity control, following the protocol described by Arahall *et al.* (2008). In both cases, PCR amplicons of MD5^T and *T. naphthalenivorans* CECT 7648^T were purified using the UltraClean PCR clean-up kit. Subsequent sequencing reactions were done on an ABI Prism 3730 automated sequencer using the Big Dye Terminator version 3.1 cycle sequencing kit, premixed format. Sequencing primers were the same as those used in the amplification reaction but diluted tenfold (5 pmol). The newly obtained sequences were compared with publicly available sequences in the GenBank/EMBL/DDBJ databases using the BLAST program (National Center for Biotechnology Information; <http://ncbi.nlm.nih.gov/>). Related sequences were further analysed using the ARB program package (Ludwig *et al.*, 2004; <http://www.arb-home.de>). Automated sequence alignments were corrected manually using the ARB_EDIT sequence editor. Alignments retrieved from the latest updates of SILVA and LTP (Pruesse *et al.*, 2007; Yarza *et al.*, 2010) were also used as references. Phylogenetic analysis using alternative treeing methods (maximum-parsimony, maximum-likelihood and distance matrix) and data subsets were performed using the appropriate ARB tools (Ludwig *et al.*, 2004). Phylogenetic analysis of the 16S rRNA gene confirmed that strain MD5^T belongs to the genus *Tropicibacter*, branching always together with *T. naphthalenivorans*.

16S rRNA gene sequence analysis showed that sequence AB302370, corresponding to the type strain *T. naphthalenivorans* C02^T, showed the highest similarity (96.9%) to the sequence of strain MD5^T, whereas *P. inhibens* T5^T exhibited 96.2% similarity, both *P. gallaeciensis* BS107^T and *P. daeponensis* TF-218^T showed 96.1% similarity and the type strains of other species with validly published names showed less than 95.9% similarity to the sequence of strain MD5^T.

Table 2. Differential characteristics between *T. multivorans* sp. nov. MD5^T and *T. naphthalenivorans* CECT 7648^T

Strains: 1, *T. multivorans* sp. nov. MD5^T; 2, *T. naphthalenivorans* CECT 7648^T. All data were obtained in this study. +, Positive; –, negative or not detected.

Characteristic	1	2
Growth at 40 °C	–	+
Specific requirement for Mg ²⁺	–	+
Assimilation of (API 20NE):		
D-Glucose	+	–
Maltose	+	–
Adipate	+	–
Malate	+	–
Enzyme activities (API ZYM)		
α-Glucosidase	+	–
β-Glucosidase	–	+
Use of sole carbon sources on BMA		
D-Ribose, D-xylose, D-mannose, cellobiose, D-mannitol, D-sorbitol, myo-inositol	+	–
Acetate, pyruvate, propionate, butyrate, malate, 3-hydroxybutyrate	+	–
L-Leucine, L-threonine, L-glutamate, L-alanine, L-arginine, L-ornithine, L-aspartate, L-histidine	+	–
Presence of cellular fatty acids		
C _{10:0} 3-OH	+	–
C _{12:1} 3-OH	–	+
C _{16:0} 2-OH	+	–

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